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Short Communication

Natural Occurrence of Indole-3-acetylaspartate and Indole-3-acetylglutamate in Cucumber Shoot Tissue[1]

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ABSTRACT

Indole-3-acetylaspartate and indole-3-acetylglutamate were isolated from 1-week-old, green cucumber shoots that had not been pretreated with auxin. Using isotope dilution techniques, we found 129 micrograms (0.42 micromoles) of indoleacetylglutamate and 33 micrograms (0.11 micromoles) of indoleacetylaspartate per kilogram of fresh tissue.

In addition to occurring in its free form, IAA is found in plants in a variety of conjugated forms: the bound auxins (4). Recently, progress has been made in the study of amide-bound auxins such as IAAsp[2], which was first reported by Andreae and Good as a metabolite of exogenous IAA (1). IAGlu is also produced from exogenous IAA in some plants (5, 10, 11). Previous work in our laboratory has shown that IAAsp and IAGlu are the sole amide-bound auxins produced in quantity when cucumber shoots are pretreated with IAA (10). Our next concern was to determine whether these compounds are also found in cucumber shoots not treated with exogenous IAA. Such natural occurrence of IAAsp has been established definitively for soybeans by Cohen (3), and somewhat less rigorously for a few other plants (see *e.g.* 9). There has been no report of the natural occurrence of IAGlu in untreated plants.

MATERIALS AND METHODS

Plant Material. Seeds of *Cucumis sativus* L. (cv Straight Eight, Burpee Seed Co.) were soaked for 1 h in tap water and sown in moist, sterilized vermiculite. Seedlings developed for 6 or 7 d in a growth chamber at 27°C on a daily photoperiodic regime of 16 h light and 8 h darkness. Light was from mixed incandescent and cool-white fluorescent sources.

Synthesis of Radioactively Labeled Amide-Bound Auxins. [^{14}C]IAAsp and [^{14}C]IAGlu were prepared from [1-^{14}C]IAA (Amersham) and di-*t*-butyl-L-aspartate or di-*t*-butyl-L-glutamate (Sigma), respectively, following the method of Cohen (2).

Isolation and Purification of Amide-Bound Auxins from Shoot Tissue. After 6 or 7 d of growth, shoots were harvested by cutting the seedlings at soil level. The tissue (500–800 g) was ground 3 times successively in a Waring Blendor with a total of 3 L of methanol. After each homogenization, the mixture was passed through eight layers of cheesecloth and then through Whatman No. 1 filter paper. The solid residues of the first two homogenizations were reextracted with methanol. After the third extraction, the residue was colorless. The green methanolic solution was kept at 4°C in the dark for 2 d. This extract was then passed through filter paper to remove precipitated material. ^{14}C labeled IAASp or IAGlu was added to the filtered solution as a marker for later isolation of native bound auxin. The volume of the solution was reduced to approximately 40 ml in a rotary evaporator. The resulting brown solution was passed through a Whatman Sep-Pak (a small plastic cartridge containing a reverse phase C_{18} stationary phase).

IAGlu was purified from such an extract of 792 g of 7-d old cucumber shoots. The first purification step was chromatography on a 33 × 3.5 cm column composed of 25 g of PVP (Aldrich), after the method of Glenn *et al.* (6). Bound auxins were eluted with 50 mM sodium phosphate buffer (pH 8.0). Active fractions (see below) were then applied to a 14 × 1.8 cm column of 5 g of DEAE-cellulose (Whatman); elution was with 10 mM Na_2SO_4 (8). The active fractions from the DEAE-cellulose column were combined, acidified to pH 2 with dilute H_3PO_4, and extracted three times with equal volumes of ethyl acetate. Volume of the combined ethyl acetate fractions was reduced under vacuum, and the concentrated material was applied to a column of silica gel (Biosil A, Bio-Rad; 65 g, 43 × 2.5 cm). Elution was with isopropanol-NH_4OH-H_2O (8:1:1, v/v). The next purification step was gel filtration on Sephadex LH-20 (Sigma; 20 g, 36 × 1.5 cm), with 50% aqueous methanol as the eluting solvent, by a modification of the technique of Cohen (2).

An aliquot of each fraction collected from the various chromatographic steps was added to 4 ml of Bray's solution and analyzed in a Beckman Series 9000 liquid scintillation counter to determine which fractions contained label from the added [^{14}C]IAGlu. All fractions containing radioactivity significantly above background were combined, reduced in volume with a rotary evaporator, and applied to the next column.

IAAsp was purified from an extract of 557 g of 6-d-old cucumber shoots. The procedure was identical to that described for IAGlu, except that the ethyl acetate extraction step preceded DEAE-cellulose chromatography rather than following it. [^{14}C] IAAsp was used as the marker.

Quantitative Analysis of Bound Auxins. Aliquots of the purified extracts were subjected to HPLC in a Varian model 5000 as described by Hollenberg *et al.* (7). We used a Varian MCH-10, 30 cm × 4 mm, reverse-phase C_{18} column equipped with a precolumn. The solvents were 1% (aqueous) glacial acetic acid (A) and acetonitrile (B). These were supplied according to the following program: pure A from 0 to 5 min, a linear gradient from 0 to 30% B from 5 to 30 min, and a linear gradient to 100% B

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[2] Abbreviations: IAAsp, indoleacetylaspartate; IAGlu, indoleacetylglutamate.

from 30 to 45 min. The flow rate was 1 ml/min. Prior to injection of samples, the column was preconditioned using a gradient from 100% B to 100% A, followed by 20 min at 100% A. Fractions were collected every 20 s, added to 4 ml of Bray's solution, and analyzed in the liquid scintillation counter. UV absorbance at 280 nm was monitored throughout the HPLC runs.

The single absorbance peaks corresponding to IAGlu and IAAsp in the purified extracts were confirmed by co-chromatography with synthetic IAGlu and IAAsp. The concentrations of bound auxins represented by the peaks of UV absorbance were determined by comparison with HPLC profiles of IAA standards. We assumed that the extinction coefficients of IAA, IAGlu, and IAAsp are equal.

RESULTS AND DISCUSSION

The levels of endogenous bound auxins were determined by isotope dilution according to the equation

$$\frac{C_o}{C_f} - 1x = y$$

where C_o is the specific radioactivity of the bound auxin added as marker, C_f the specific radioactivity of the recovered bound auxin, x the amount of bound auxin added as marker, and y the amount of endogenous bound auxin. The quantities are presented in Table I.

Thus, 7-d-old cucumber shoots contained 129 μg (0.42 μmol) of IAGlu per kg of tissue, and 6-d-old shoots contained 33 μg (0.11 μmol) of IAAsp per kg of tissue. The values are tentative, being based on single determinations. These values, for growing shoot tissue, may be compared with that reported by Cohen (3) for IAAsp in dry soybean seeds (10 μmol/kg).

Table I. *IAAsp and IAGlu Extraction Data*

	Quantity[a]			
	C_o	C_f	x	y
	μCi/mg		μg	
IAAsp	264	81.2	8.2	18.5
IAGlu	258	38.9	8.2	103

[a] See isotope dilution equation.

This is the first report of the occurrence of IAGlu in plant tissue not pretreated with IAA. The two amide-bound auxins now known to occur naturally in untreated plants are IAGlu and IAAsp.

We have previously shown that IAAsp and IAGlu are the only two amide-bound auxins formed when cucumber shoots are pretreated with IAA (at least in the absence of other treatments). In further studies of the physiological roles of bound auxins in cucumber shoots, it is reasonable to assume that IAAsp and IAGlu are the major amide-bound auxins in this plant.

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